

CLAIMS

- 5 1. A strain of *M. bovis* BCG or *M. microti*, wherein said strain has integrated all or part of the fragment, named RD1-2F9, of 31808 pb of DNA originating from *Mycobacterium tuberculosis* or any virulent member of the *Mycobacterium tuberculosis* complex (*M. africanum*, *M. bovis*, *M. canettii*), as shown in SEQ ID No 1 and which is responsible for enhanced immunogenicity and increased persistence
10 of BCG to the tubercle bacilli.
2. A strain of *M. bovis* BCG or *M. microti* according to claim 1, wherein said strain has integrated all or part of the fragment of DNA originating from *Mycobacterium tuberculosis* or any virulent member of the *Mycobacterium tuberculosis* complex (*M. africanum*, *M. bovis*, *M. canettii*) as shown in SEQ ID No 2 responsible for
15 enhanced immunogenicity and increased persistence of BCG to the tubercle bacilli.
3. A strain of *M. bovis* BCG or *M. microti* according to claim 1, wherein said strain has integrated all or part of the fragment of DNA originating from *Mycobacterium tuberculosis* or any virulent member of the *Mycobacterium tuberculosis* complex (*M. africanum*, *M. bovis*, *M. canettii*) as shown in SEQ ID No 3 responsible for
20 enhanced immunogenicity and increased persistence of BCG to the tubercle bacilli.
4. A strain according to claim 1 which has integrated a portion of DNA originating from *Mycobacterium tuberculosis* or any virulent member of the *Mycobacterium tuberculosis* complex (*M. africanum*, *M. bovis*, *M. canettii*), which comprises at least one, two, three or more gene(s) selected from Rv3861 (SEQ ID No 4), Rv3862
25 (SEQ ID No 5), Rv3863 (SEQ ID No 6), Rv3864 (SEQ ID No 7), Rv3865 (SEQ ID

- No 8), Rv3866 (SEQ ID No 9), Rv3867 (SEQ ID No 10), Rv3868 (SEQ ID No 11), Rv3869 (SEQ ID No 12), Rv3870 (SEQ ID No 13), Rv3871 (SEQ ID No 14), Rv3872 (SEQ ID No 15, mycobacterial PE), Rv3873 (SEQ ID No 16, PPE), Rv3874 (SEQ ID No 17, CFP-10), Rv3875 (SEQ ID No 18, ESAT-6), Rv3876 (SEQ ID No 19), Rv3877 (SEQ ID No 20), Rv3878 (SEQ ID No 21), Rv3879 (SEQ ID No 22), Rv3880 (SEQ ID No 23), Rv3881 (SEQ ID No 24), Rv3882 (SEQ ID No 25), Rv3883 (SEQ ID No 26), Rv3884 (SEQ ID No 27) and Rv3885 (SEQ ID No 28).
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5. A strain according to claim 1 which has integrated a portion of DNA originating from *Mycobacterium tuberculosis* or any virulent member of the *Mycobacterium tuberculosis* complex (*M. africanum*, *M. bovis*, *M. canettii*), which comprises at least one, two, three or more gene(s) selected from Rv3867 (SEQ ID No 10), Rv3868 (SEQ ID No 11), Rv3869 (SEQ ID No 12), Rv3870 (SEQ ID No 13), Rv3871 (SEQ ID No 14), Rv3872 (SEQ ID No 15, mycobacterial PE), Rv3873 (SEQ ID No 16, PPE), Rv3874 (SEQ ID No 17, CFP-10), Rv3875 (SEQ ID No 18, ESAT-6), Rv3876 (SEQ ID No 19) and Rv3877 (SEQ ID No 20).
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6. A strain according to claim 1 which has integrated a portion of DNA originating from *Mycobacterium tuberculosis* or any virulent member of the *Mycobacterium tuberculosis* complex (*M. africanum*, *M. bovis*, *M. canettii*), which comprises at least one, two, three or more gene(s) selected from Rv3872 (SEQ ID No 15, mycobacterial PE), Rv3873 (SEQ ID No 16, PPE), Rv3874 (SEQ ID No 17, CFP-10) and Rv3875 (SEQ ID No 18, ESAT-6).
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7. A strain according to claim 1 which has integrated a portion of DNA originating from *Mycobacterium tuberculosis* or any virulent member of the *Mycobacterium tuberculosis* complex (*M. africanum*, *M. bovis*, *M. canettii*), which comprises at least four genes selected from Rv3861 (SEQ ID No 4), Rv3862 (SEQ ID No 5), Rv3863 (SEQ ID No 6), Rv3864 (SEQ ID No 7), Rv3865 (SEQ ID No 8), Rv3866
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- (SEQ ID No 9), Rv3867 (SEQ ID No 10), Rv3868 (SEQ ID No 11), Rv3869 (SEQ ID No 12), Rv3870 (SEQ ID No 13), Rv3871 (SEQ ID No 14), Rv3872 (SEQ ID No 15, mycobacterial PE), Rv3873 (SEQ ID No 16, PPE), Rv3874 (SEQ ID No 17, CFP-10), Rv3875 (SEQ ID No 18, ESAT-6), Rv3876 (SEQ ID No 19), Rv3877 (SEQ ID No 20), Rv3878 (SEQ ID No 21), Rv3879 (SEQ ID No 22), Rv3880 (SEQ ID No 23), Rv3881 (SEQ ID No 24), Rv3882 (SEQ ID No 25), Rv3883 (SEQ ID No 26), Rv3884 (SEQ ID No 27) and Rv3885 (SEQ ID No 28)., provided that it comprises Rv3874 (SEQ ID No 17, CFP-10) and/or Rv3875 (SEQ ID No 18, ESAT-6).
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- 10 8. A strain according to claim 1 which has integrated a portion of DNA originating from *Mycobacterium tuberculosis* or any virulent member of the *Mycobacterium tuberculosis* complex (*M. africanum*, *M. bovis*, *M. canettii*), which comprises at least Rv3871 (SEQ ID No 14), Rv3875 (SEQ ID No 18, ESAT-6) and Rv3876 (SEQ ID No 19).
- 15 9. A strain according to claim 1 which has integrated a portion of DNA originating from *Mycobacterium tuberculosis* or any virulent member of the *Mycobacterium tuberculosis* complex (*M. africanum*, *M. bovis*, *M. canettii*), which comprises at least Rv3871 (SEQ ID No 14), Rv3875 (SEQ ID No 18, ESAT-6) and Rv3877 (SEQ ID No 20).
- 20 10. A strain according to claim 1 which has integrated a portion of DNA originating from *Mycobacterium tuberculosis* or any virulent member of the *Mycobacterium tuberculosis* complex (*M. africanum*, *M. bovis*, *M. canettii*), which comprises at least Rv3871 (SEQ ID No 14), Rv3875 (SEQ ID No 18, ESAT-6), Rv3876 (SEQ ID No 19) and Rv3877 (SEQ ID No 20).

11. A strain according to one of claims 8 to 10 which has integrated a portion of DNA originating from *Mycobacterium tuberculosis* or any virulent member of the *Mycobacterium tuberculosis* complex (*M. africanum*, *M. bovis*, *M. canettii*), which further comprises Rv3874 (SEQ ID No 17, CFP-10).
- 5 12. A strain according to one of claims 8 to 11 which has integrated a portion of DNA originating from *Mycobacterium tuberculosis* or any virulent member of the *Mycobacterium tuberculosis* complex (*M. africanum*, *M. bovis*, *M. canettii*), which further comprises Rv3872 (SEQ ID No 15, mycobacterial PE).
- 10 13. A strain according to one of claims 8 to 12 which has integrated a portion of DNA originating from *Mycobacterium tuberculosis* or any virulent member of the *Mycobacterium tuberculosis* complex (*M. africanum*, *M. bovis*, *M. canettii*), which further comprises Rv3873 (SEQ ID No 16, PPE).
- 15 14. A strain according to one of claims 8 to 13 which has integrated a portion of DNA originating from *Mycobacterium tuberculosis* or any virulent member of the *Mycobacterium tuberculosis* complex (*M. africanum*, *M. bovis*, *M. canettii*), which further comprises at least one, two, three or four gene(s) selected from Rv3861 (SEQ ID No 4), Rv3862 (SEQ ID No 5), Rv3863 (SEQ ID No 6), Rv3864 (SEQ ID No 7), Rv3865 (SEQ ID No 8), Rv3866 (SEQ ID No 9), Rv3867 (SEQ ID No 10), Rv3868 (SEQ ID No 11), Rv3869 (SEQ ID No 12), Rv3870 (SEQ ID No 13),
20 Rv3878 (SEQ ID No 21), Rv3879 (SEQ ID No 22), Rv3880 (SEQ ID No 23), Rv3881 (SEQ ID No 24), Rv3882 (SEQ ID No 25), Rv3883 (SEQ ID No 26), Rv3884 (SEQ ID No 27) and Rv3885 (SEQ ID No 28).
- 25 15. A strain according to claim 1 which has integrated a portion of DNA originating from *Mycobacterium tuberculosis* or any virulent member of the *Mycobacterium*

tuberculosis complex (*M. africanum*, *M. bovis*, *M. canettii*), which comprises Rv3875 (SEQ ID No 18, ESAT-6).

16. A strain according to claim 1 which has integrated a portion of DNA originating from *Mycobacterium tuberculosis* or any virulent member of the *Mycobacterium tuberculosis* complex (*M. africanum*, *M. bovis*, *M. canettii*), which comprises Rv3874 (SEQ ID No 17, CFP-10).
17. A strain according to claim 1 which has integrated a portion of DNA originating from *Mycobacterium tuberculosis* or any virulent member of the *Mycobacterium tuberculosis* complex (*M. africanum*, *M. bovis*, *M. canettii*), which comprises both Rv3875 (SEQ ID No 18, ESAT-6) and Rv3874 (SEQ ID No 17, CFP-10).
18. A strain according to one of claims 4 to 17, wherein the coding sequence of the integrated gene is in frame with its natural promoter or with an exogenous promoter, such as a promoter capable of directing high level of expression of said coding sequence.
19. A strain according to one of claims 4 to 17, wherein the said integrated gene is mutated so as to maintain the improved immunogenicity while decreasing the virulence of the strain.
20. A strain according to claim 18 or 19, wherein said strain only carries parts of the genes coding for ESAT-6 or CFP-10 in a mycobacterial expression vector under the control of a promoter, more particularly an hsp60 promoter.
21. A strain according to claim 18, wherein said strain carries at least one portion of the *esat-6* gene that codes for immunogenic 20-mer peptides of ESAT-6 active as T-cell epitopes.

22. A strain according to claim 19, wherein the *esat-6* encoding gene is altered by directed mutagenesis in a way that most of the immunogenic peptides of ESAT-6 remain intact, but the biological functionality of ESAT-6 is lost.

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23. A strain according to claim 19, wherein the CFP-10 encoding gene is altered by directed mutagenesis in a way that most of the immunogenic peptides of CFP-10 remain intact, but the biological functionality of CFP-10 is lost.

10 24. *M. bovis* BCG::RD1 strains which have integrated a cosmid herein referred to as RD1-2F9 and RD1-AP34 contained in the *E. coli* strains deposited at the CNCM under the accession number I-2831 and I-2832 respectively.

15 25. *M. bovis* BCG::RD1 strain which has integrated the insert of the cosmid RD1-AP34 which corresponds to the 3909 bp fragment of the *M. tuberculosis* H37Rv genome from region 4350459 bp to 4354367 bp cloned as shown in SEQ ID No 3.

20 26. *M. bovis* BCG::RD1 strain which has integrated the insert of the cosmid RD1-2F9 (~ 32 kb) that covers the region of the *M. tuberculosis* genome AL123456 from ca 4337 kb to ca. 4369 kb as shown in SEQ ID No 1.

25 27. *M. microti*::RD1 strain which has integrated the insert of the cosmid RD1-AP34 which corresponds to the 3909 bp fragment of the *M. tuberculosis* H37Rv genome from region 4350459 bp to 4354367 bp cloned as shown in SEQ ID No 3).

28. *M. microti*::RD1 strain which has integrated the insert of the cosmid RD1-2F9 (~ 32 kb) that covers the region of the *M. tuberculosis* genome AL123456 from ca 4337 kb to ca. 4369 kb as shown in SEQ ID No 1.

29. A method for preparing and selecting improved *M. bovis* BCG or *M. microti* strains defined in any one of claims 1 to 28 comprising a step consisting of modifying said strains by insertion, deletion or mutation in the integrated portion of DNA originating from *Mycobacterium tuberculosis* or any virulent member of the *Mycobacterium tuberculosis* complex (*M. africanum*, *M. bovis*, *M. canettii*), more particularly in the *esat-6* or *CFP-10* gene, said method leading to strains that are less virulent for immuno-depressed individuals.
30. A cosmid or a plasmid comprising a portion of DNA originating from *Mycobacterium tuberculosis* or any virulent member of the *Mycobacterium tuberculosis* complex (*M. africanum*, *M. bovis*, *M. canettii*), said portion of DNA comprising at least one, two, three or more gene(s) selected from Rv3861 (SEQ ID No 4), Rv3862 (SEQ ID No 5), Rv3863 (SEQ ID No 6), Rv3864 (SEQ ID No 7), Rv3865 (SEQ ID No 8), Rv3866 (SEQ ID No 9), Rv3867 (SEQ ID No 10), Rv3868 (SEQ ID No 11), Rv3869 (SEQ ID No 12), Rv3870 (SEQ ID No 13), Rv3871 (SEQ ID No 14), Rv3872 (SEQ ID No 15, mycobacterial PE), Rv3873 (SEQ ID No 16, PPE), Rv3874 (SEQ ID No 17, CFP-10), Rv3875 (SEQ ID No 18, ESAT-6), Rv3876 (SEQ ID No 19), Rv3877 (SEQ ID No 20), Rv3878 (SEQ ID No 21), Rv3879 (SEQ ID No 22), Rv3880 (SEQ ID No 23), Rv3881 (SEQ ID No 24), Rv3882 (SEQ ID No 25), Rv3883 (SEQ ID No 26), Rv3884 (SEQ ID No 27) and Rv3885 (SEQ ID No 28).
31. A cosmid or a plasmid comprising a portion of DNA originating from *Mycobacterium tuberculosis* or any virulent member of the *Mycobacterium tuberculosis* complex (*M. africanum*, *M. bovis*, *M. canettii*), said portion of DNA comprising at least one, two, three or more gene(s) selected from Rv3867 (SEQ ID No 10), Rv3868 (SEQ ID No 11), Rv3869 (SEQ ID No 12), Rv3870 (SEQ ID No 13), Rv3871 (SEQ ID No 14), Rv3872 (SEQ ID No 15, mycobacterial PE), Rv3873

(SEQ ID No 16, PPE), Rv3874 (SEQ ID No 17, CFP-10), Rv3875 (SEQ ID No 18, ESAT-6), Rv3876 (SEQ ID No 19) and Rv3877 (SEQ ID No 20).

32. A cosmid or a plasmid comprising a portion of DNA originating from *Mycobacterium tuberculosis* or any virulent member of the *Mycobacterium tuberculosis* complex (*M. africanum*, *M. bovis*, *M. canettii*), said portion of DNA comprising at least one gene selected from Rv3871 (SEQ ID No 14), Rv3872 (SEQ ID No 15, mycobacterial PE), Rv3873 (SEQ ID No 16, PPE), Rv3874 (SEQ ID No 17, CFP-10), Rv3875 (SEQ ID No 18, ESAT-6) and Rv3876 (SEQ ID No 12).
33. A cosmid or a plasmid according to any of claims 30 to 32 comprising Rv3874 encoding CFP-10, Rv3875 encoding ESAT-6 or both or a part of them.
34. A cosmid or a plasmid according to any of claims 30 to 33 comprising a mutated gene selected among Rv3861 to Rv3885.
35. A cosmid or a plasmid according to claim 30 which comprises at least four genes selected from Rv3861 (SEQ ID No 4), Rv3862 (SEQ ID No 5), Rv3863 (SEQ ID No 6), Rv3864 (SEQ ID No 7), Rv3865 (SEQ ID No 8), Rv3866 (SEQ ID No 9), Rv3867 (SEQ ID No 10), Rv3868 (SEQ ID No 11), Rv3869 (SEQ ID No 12), Rv3870 (SEQ ID No 13), Rv3871 (SEQ ID No 14), Rv3872 (SEQ ID No 15, mycobacterial PE), Rv3873 (SEQ ID No 16, PPE), Rv3874 (SEQ ID No 17, CFP-10), Rv3875 (SEQ ID No 18, ESAT-6), Rv3876 (SEQ ID No 19), Rv3877 (SEQ ID No 20), Rv3878 (SEQ ID No 21), Rv3879 (SEQ ID No 22), Rv3880 (SEQ ID No 23), Rv3881 (SEQ ID No 24), Rv3882 (SEQ ID No 25), Rv3883 (SEQ ID No 26), Rv3884 (SEQ ID No 27) and Rv3885 (SEQ ID No 28), provided that it comprises Rv3874 (SEQ ID No 17, CFP-10) and/or Rv3875 (SEQ ID No 18, ESAT-6)).
36. A cosmid or a plasmid according to claim 30 which comprises at least Rv3871 (SEQ ID No 14), Rv3875 (SEQ ID No 18, ESAT-6) and Rv3876 (SEQ ID No 19)).

37. A cosmid or a plasmid according to claim 30 which comprises at least Rv3871 (SEQ ID No 14), Rv3875 (SEQ ID No 18, ESAT-6) and Rv3877 (SEQ ID No 20).
38. A cosmid or a plasmid according to claim 30 comprising at least Rv3871 (SEQ ID No 14), Rv3875 (SEQ ID No 18, ESAT-6), Rv3876 (SEQ ID No 19) and Rv3877 (SEQ ID No 20).
39. A cosmid or a plasmid according to one of claims 36 to 38 which further comprises Rv3872 (SEQ ID No 15, mycobacterial PE) Rv3873 (SEQ ID No 16, PPE) Rv3874 (SEQ ID No 17, CFP-10).
40. A cosmid or a plasmid according to one of claims 36 to 38 which further comprises at least one, two, three or four gene(s) selected from Rv3861 (SEQ ID No 4), Rv3862 (SEQ ID No 5), Rv3863 (SEQ ID No 6), Rv3864 (SEQ ID No 7), Rv3865 (SEQ ID No 8), Rv3866 (SEQ ID No 9), Rv3867 (SEQ ID No 10), Rv3868 (SEQ ID No 11), Rv3869 (SEQ ID No 12), Rv3870 (SEQ ID No 13), Rv3878 (SEQ ID No 21), Rv3879 (SEQ ID No 22), Rv3880 (SEQ ID No 23), Rv3881 (SEQ ID No 24), Rv3882 (SEQ ID No 25), Rv3883 (SEQ ID No 26), Rv3884 (SEQ ID No 27) and Rv3885 (SEQ ID No 28).
41. A cosmid herein referred as RD1-2F9 and RD1-AP34 contained in the *E. coli* strains deposited at the CNCM under the accession number I-2831 and I-2832 respectively.
42. Use of a cosmid or a plasmid according to one of claims 30 to 41 for transforming *M. bovis* BCG or *M. microti*.
43. A pharmaceutical composition comprising a strain according to one of claims 1 to 27 and a pharmaceutically acceptable carrier.

44. A pharmaceutical composition according to claim 40 containing suitable pharmaceutically-acceptable carriers comprising excipients and auxiliaries which facilitate processing of the living vaccine into preparations which can be used pharmaceutically.
- 5 45. A pharmaceutical composition according to claim 40 or 41 which is suitable for intravenous or subcutaneous administration.
46. A vaccine comprising a strain according to one of claims 1 to 28 and a suitable carrier.
47. A product comprising a strain according to one of claims 1 to 28 and at least one
10 protein selected from ESAT-6 and CFP-10 or epitope derived thereof for a separate, simultaneous or sequential use for treating tuberculosis.
48. The use of a strain according to one of claims 1 to 28 for preparing a medicament or a vaccine for preventing or treating tuberculosis.
49. The use of a strain according to one of claims 1 to 28 as an
15 adjuvant/immunomodulator for preparing a medicament for the treatment of superficial bladder cancer.
50. A method for the identification at the species level of members of the *M. tuberculosis* complex by means of markers for RD1^{mic} and RD5^{mic} as molecular diagnostic test.
- 20 51. A method according to claim 50 comprising the use of a primer selected from :
primer esat-6F GTCACGTCCATTTCATTCCT (SEQ ID No 32),
primer esat-6R ATCCCAGTGACGTTGCCTT) (SEQ ID No 33),

primer RD1^{mic} flanking region F GCAGTGCAAAGGTGCAGATA (SEQ ID No 34),
 primer RD1^{mic} flanking region R GATTGAGACACTTGCCACGA (SEQ ID No 35),
 primer RD5^{mic} flanking region F GAATGCCGACGTCATATCG (SEQ ID No 39),
 primer RD5^{mic} flanking region R CGGCCACTGAGTTCGATTAT (SEQ ID No 40)
 5 and the complementary sequences of said primers.

52. A diagnostic kit for the identification at the species level of members of the *M. tuberculosis* complex comprising DNA probes and primers specifically hybridizing to a DNA portion of the RD1 or RD5 region of *M. tuberculosis*, more particularly probes hybridizing under stringent conditions to a gene selected from Rv3871 (SEQ ID No 14),
 10 Rv3872 (SEQ ID No 15, mycobacterial PE), Rv3873 (SEQ ID No 16, PPE), Rv3874 (SEQ ID No 17, CFP-10), Rv3875 (SEQ ID No 18, ESAT-6), Rv3876 (SEQ ID No 19) and Rv3877 (SEQ ID No 20), preferably CFP-10 and ESAT-6.

53. A diagnostic kit according to claim 52 comprising a probe or primer selected from :
 primer esat-6F GTCACGTCCATTCATTCCCT (SEQ ID No 32),
 15 primer esat-6R ATCCCAGTGACGTTGCCTT) (SEQ ID No 33),
 primer RD1^{mic} flanking region F GCAGTGCAAAGGTGCAGATA (SEQ ID No 34),
 primer RD1^{mic} flanking region R GATTGAGACACTTGCCACGA (SEQ ID No 35),
 primer RD5^{mic} flanking region F GAATGCCGACGTCATATCG (SEQ ID No 39),
 primer RD5^{mic} flanking region R CGGCCACTGAGTTCGATTAT (SEQ ID No 40)
 20 and the complementary sequences of said primers.

54. A diagnostic kit for the identification at the species level of members of the *M. tuberculosis* complex comprising at least one, two, three or more antibodies directed to mycobacterial PE, PPE, CFP-10, ESAT-6.

55. A diagnostic kit according to claim 54 wherein it comprises antibodies directed to CFP-10 and ESAT-6.

56. Virulence markers associated with RD1 and/or RD5 regions of the genome of *M. tuberculosis* or a part of these regions.

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57. The use of a strain according to one of claims 1 to 28 as a carrier for the expression of a molecule or an heterologous antigen that are of therapeutic or prophylactic interest.

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58. A purified nucleic acid corresponding to the *Mycobacterium* DNA inserted in a cosmid according to any of claims 30 to 41.

59. The purified nucleic acid according to claim 58 which corresponds to the insert of cosmid RD1-2F9 or cosmid RD1-AP34.